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ACETYLCHOLINESTERASE AND NERVE CONDUCTION

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According to the prevailing theory, the propagation of a nerve impulse is dependent upon (1) the release of acetylcholine near a receptor protein, (2) the depolarization of the excitable membrane and (3) the rapid hydrolysis of acetylcholine by the closely situated enzyme, acetylcholinesterase, thereby restoring the membrane to a polarized state. This theory is based primarily upon results of *in vitro* studies conducted chiefly by Nachmansohn and collegues (1).

It follows that in the presence of a cholinesterase inhibitor, acetylcholine would be allowed to accumulate to such a high concentration as to cause blockade of nerve conduction. This has indeed been shown to be the case in *in vitro* preparations such as the single giant axon of the squid (2), the lobster walking-leg nerve bundle (3) and in the isolated frog sciatic nerve (4). In all of these preparations the concentrations of inhibitors required to inhibit conduction were extremely large. Murtha et. al. (5) reported that following the administration of Tetraethylpyrophosphate (1.6 mg/kg) the nerve action potential of the *in vivo* cat sciatic gastrocnemius-soleus muscle preparation was greatly reduced in amplitude or completely abolished simultaneously with the mechanical response of the muscle at a time when considerable amounts of active enzyme was still present in the nerve.

Recently, Sechzer (6) found no lasting effect of several organophosphorous compounds, including 217 AO, 217 MI and DFP on nerve conduction in the intact sciatic nerve of the cat. He concluded that even though these compounds are lipid soluble to varying degrees the possibility exists that they did not reach the site of action in sufficient concentration to block conduction. Crescitelli et. al. (4), using bullfrog nerves *in vivo*, showed that nerves essentially free of acetylcholinesterase conduct impulses in the same

manner as normal nerves in response to both single and repetitive stimuli. He concluded that cholinesterase is not essential for nerve conduction.

In view of these conflicting reports, it was felt that the problem merits re-investigation since, from the standpoint of military research and public health, all efforts have been concerned only with the neuromuscular effects of organophosphorous anticholinesterase compounds and insectisides and all therapeutic measures have considered only the "biochemical lesion" produced at the neuromuscular junction.

This study was initiated in order to investigate the effects of several potent anticholinesterase compounds on conduction in peripheral nerve. In view of the crucial importance of any relationship existing between the events of conduction and enzyme activity, it was desirable to use an in vivo mammalian preparation. It will be shown that such nerves are able to conduct impulses to both single and repetitive stimuli following the intravenous injection of several elthal doses of different types of lipid soluble acetylcholinesterase inhibitors.

PROCEDURES

Anesthetized cats (Dial-Urethane i. p.) weighing between 2 and 3 kg were used in all experiments. The lower lumbar and upper sacral segments of the spinal cord were exposed. A ventral root filament of L_7 was severed close to its outflow from the cord and the distal end tied with a loop of silk thread and lifted onto bipolar silver recording electrodes. Dorsal roots of L_6, L_7, S_1 and S_2 segments were severed. The left sciatic nerve was exposed in the popliteal region of the leg and lifted onto a pair of silver stimulating electrodes. Both segments of nerve were kept moist under pools of extra heavy mineral oil created by typing and lifting the edges of skin in the lower back region. Muscle action potentials were recorded by a concentric needle electrode inserted directly into the gastrocnemius muscle. The indirectly evoked muscle and nerve action potentials were displayed on a Tetronix Type - 502 dual beam cathode-ray oscilloscope and photographed from the screen by an attached Grass Model C4D kymograph camera. Supramaximal shocks were delivered to the sciatic nerve at frequencies of 1 cps and 40 cps (duration = 0.05 msec.) by a Grass Model S4B stimulator. The femoral vein was cannulated by which all injections were made. All animals were atropinized (2 mg/kg) prior to the injection of the anticholinesterase agents. Artificial respiration was administered through a tracheal cannula as needed. The right sciatic nerve was removed prior to any injection and partially desheathed for determination of control cholinesterase activity. The left sciatic

nerve was removed at the end of the experiment for comparative enzyme determination. Enzyme activity was determined by both a modified Hestrin colorimetric technique (7) and the histochemical technique of Karnovsky (8).

Drugs employed in this study included: Tetraethyl-pyrophosphate (TEPP), Di-isopropyl Fluorophosphate (DFP), Pinacolyl methylphosphonofluoridate (Soman), Isopropyl methylphosphonofluoridate (Sarin), Monoisonitrosoacetone (MINA), d-Tubocurarine and Atropine sulfate. All drug concentrations are expressed in mg/kg body weight, unless otherwise stated.

RESULTS

The in vivo administration of a large dose of anticholinesterase agent should, theoretically, result in the accumulation of acetylcholine with continuous depolarization of the excitable membrane causing blockade of nerve conduction. Figure 1 shows the effect of Soman (0.15 mg/kg) on the electrically evoked nerve and muscle action potentials. At the left is shown control responses to 1 cps and 40 cps stimulation of the sciatic nerve. In this and the figures to follow the nerve action potential appears as the first spike. Twenty seconds after the Soman injection the muscle action potential had decreased by about 50 per cent. The waves in the base line of the traces are due to the general muscle fasciculations in response to the anticholinesterase. One minute post injection the muscle action potential is completely blocked, while no change appears in the nerve action potential. However, after approximately one hour, and with still no alteration in the nerve action potential, d-Tubocurarine (0.5 mg/kg) was injected. Within minutes the muscle action potential has returned to about 40 percent of control height and forty minutes after the curare injection the muscle action potential had returned to about control height. The recovery is due to antagonism of the blockade produced by the accumulated acetylcholine at the neuromuscular junction. This effect of curare is only temporary since blockade re-occurs after several hours.

After the return of muscle function acetylcholine (0.0025 mg/kg) was injected intravenously and, as seen on the lower part of Figure 1, abolished the muscle action potential while showing no effect on that of the nerve.

Rosenberg and Podleski (9) found that following pre-treatment of isolated squid axons with snake venoms to increase penetration, both acetylcholine and curare reversibly blocked nerve conduction. In our experiments, curare showed no effect on the nerve action potential even when administered prior to the anticholinesterase in a concentration of 10 LD_{50}.

The effect of intravenously administered Sarin on electrically evoked nerve and muscle action potentials is shown in Figure 2. Control responses to single (1 cps) and 40 cps) stimuli are shown at the upper part of the figure. Fifteen seconds after injecting 0.2 mg/kg of Sarin the muscle action potential was reduced to less than 20 per cent of the control and within 30 seconds was completely abolished. No effect was seen on the nerve action potential. Recordings taken 40 and 75 minutes after Sarin administration reveal the recovery of the muscle response to single stimuli. However, at this time, the muscle was unable to give a sustained response to high-frequency repetitive stimuli. This is not shown on the figure. Monoisonitrosoacetone (MINA) 86 mg/kg, one of the more potent oximes in reactivating inhibited acetylcholinesterase, was injected into the Sarin poisoned animal. No alteration was observed in the responses of either muscle or nerve to 1 cps stimulation recorded five minutes after the injection. At the bottom of Figure 2 can be seen the responses of both nerve and muscle to high-frequency (40 cps) stimulation at 30 and 60 minutes following the injection of MINA. The ability of the muscle to give a sustained response is restored.

Figure 3 shows the results obtained by injecting Tetraethylpyrophosphate (1.5 mg/kg). Control responses to both single and repetitive stimulation before injecting TEPP are shown on the upper part of the figure. The recording taken two minutes after the injection shows the decline of the muscle action potential. Four minutes after the injection complete neuromuscular block had occurred. Again, no effect was observed on the nerve action potential. Within one hour the muscle action potential had returned to control height. However, the muscle was unable to give a sustained response to high-frequency stimulation, as shown on the right. Curare (0.2 mg/kg) abolished the muscle potential again, but not the nerve potential, as shown at the bottom of the figure. Four hours later, the muscle action potential had returned to its control height.

On Figure 4 is shown the effect of DFP on the electrically evoked nerve and muscle action potentials. Responses to 1 cps stimulation are shown on the upper part of the figure. Recordings taken five and ten minutes following the injection of 16 mg/kg DFP show the declining muscle action potential without any alteration of the nerve action potential. Thirty minutes after the injection the muscle action potential had returned almost to control amplitude. Following the injection of d-Tubocurarine (0.15 mg/kg) the muscle action potential was abolished and within ninety minutes had returned. High-frequency stimulation one hour and forty minutes after curare shows the muscle regaining its ability to give a sustained response. The recording taken two minutes later shows both muscle and nerve potentials to be at the control height. After the curare effect has subsided neuromuscular block re-appears. Thus, curare

only temporarily improves neuromuscular transmission following anticholinesterase poisoning.

The acetylcholinesterase enzyme activity of the sciatic nerves, as determined by both the histochemical and colorimetric techniques, following the intravenous administration of each of the inhibitors was reduced to less than 10 per cent of the control value. In Figure 5 is shown a photomicrograph of a longitudinal section of normal sciatic nerve. Cholinesterase appears black. Figure 6 shows a photomicrograph of a longitudinal section of sciatic nerve following the administration of Soman (0.15 mg/kg). Very few areas of enzyme localization appear. A transverse section of normal nerve is illustrated in Figure 7. All the black areas represent cholinesterase. The photomicrograph of the transverse section in Figure 8 was taken after poisoning the animal with Soman (0.15 mg/kg) and shows that the enzyme is almost completely inhibited.

DISCUSSION

The results presented here, using the cat's sciatic nerve preparation as an indication of the functional integrity of peripheral nerve after poisoning with several organophosphorous anticholinesterase compounds, indicate that the animal is able to tolerate huge doses (up to 50 LD_{50}) without any apparent impairment of the nerve action potential, in spite of almost complete inactivation of its cholinesterase activity. However, the simultaneously recorded muscle action potential was completely abolished within seconds (Soman, Sarin) or minutes (DFP, TEPP) after the injections. The failure of these compounds to affect nerve conduction has been explained by some investigators (1) as due to their inability to penetrate lipid barriers surrounding the axon. However, all these compounds have been shown to penetrate lipid layers to varying degrees. It is reasonable to assume that such compounds are carried to the active sites via the blood in much the same way as other substances in blood. Evidence that these inhibitors reach the site of action is shown by the inactivation of nerve cholinesterase activity by more than 90 per cent.

It has been argued that even when using the best available technique for the determination of cholinesterase, minute quantities of enzyme escape detection because existing procedures are not sensitive enough. These quantities are said to be quite sufficient to hydrolyze the substrate present. This postulation is open to question since the absolute minimum level of acetylcholinesterase compatible with normal nerve conduction has not been reported. However, it seems reasonable to expect that inactivation of the enzyme by 90 - 95 per cent of normal would produce at least a slight alteration in the recorded nerve action potential (i.e. if the enzyme is

essential for nerve conduction. Bullock et. al. states that conduction fails when the enzyme activity drops to about 20% of its initial value.

The fact that no alterations occurred in the evoked nerve action potential might be explained by the following: (1) The axon retained functional enzyme in amounts sufficient to hydrolyze the substrate. This would mean that the axon possess some barrier to the agents which is non-existant or more easily penetrable at the motor nerve terminal, (2) The acetylcholinesterase is not as important in nerve conduction as it has been thought to be. It is entirely possible that the block of nerve conduction which is produced in vitro by the local application of various anticholinesterase agents in high concentration does not result from the anticholinesterase action of these agents.

Wright (10) has found that physostigmine (0.005 - 0.08 M) caused reversible block of conduction in isolated frog sciatic nerve and rabbit tibial nerve. From the data obtained he concludes that the blocking action cannot be due to acetylcholine accumulation by the anticholinesterase activity of physostigmine, but rather is due to a change in the ion transport across the nerve membrane; specifically, the inactivation of the "sodium mechanism". This could well be the explanation for conduction block by other cholinesterase inhibitors observed in in vitro preparations by many investigators.

SUMMARY

An attempt has been made to determine the effects of several anticholinesterase compounds on the electrically evoked nerve and muscle action potentials of the cat. It has been shown that nerves essentially devoid of detectable enzyme are able to conduct impulses without any impairment. The characteristic effects produced by these inhibitors at the neuromuscular junction have been observed. The conclusion is reached that in motor nerve fibers there is apparently no correlation between the magnitude and amplitude of the action potential acetylcholinesterase activity as determined in homogenized nerve and histochemically. It appears than, from the data presented here, the only "biochemical lesion" produced by organophosphorous anticholinesterase compounds and insecticides that need be of concern in therapeutic procedures are located at junctions (neuromuscular, synapses, ganglia, etc.) There are apparently no effects on intra-axonal conduction.

REFERENCES

1. Nachnamsohn, D. The Chemical and Molecular Basis of Nerve Activity. Academic Press. (1959).

2. Dettbarn, W. D. and Hoskin, F. C. G. Electrical and Esterase Activity In Axons. Biochemica Acta. 62: 566 (1962)

3. Dettbarn, W. D. and Davis, F. A. Effects of Acetylcholine on Axonal Conduction of Lobster Nerve. Biochem. et Biophysica Acta. 66: 397 (1963).

4. Crescitelli, F., Koelle, G. B. and Gilman, A. Transmission of Impulses in Peripheral Nerve Treated with Diisopropyl Fluorophosphate (DFP). J. Neurophysiol. 9:241 (1962).

5. Murtha, E. F., McNamara, B. P., Edberg, L. J., Bergner, A. D.B. and Wills, J. H. Studies on the Pharmacology of Tetraethylpyrophosphate. J. Pharm. Exp Therap 115: 291 (1955).

6. Sechzer, P. H. The effect of Organophosphates on Nerve Conduction. Arch. Intern. de Pharmacodyn. et de Therap. 157: 432 (1965).

7. Fleisher, J. H., Pope, E. J., and Spear, S. F. Determination of Red Blood Cell Cholinesterase Activity in the Whole Blood. A. M. A. Arch. of Industrial Health 11: 332 (1955).

8. Karnovsky, M. J. and Roots, L. A "Direct - Coloring" Thiocholine Method for Cholinesterases. J. Histochem. Cytochem. 12: 219 (1964).

9. Rosenberg, P. and Podleski, T. R. Block of Conduction by Acetylcholine and d-Tubocurarine after Treatment of Squid Axon with Cottonmouth Moccasin Venom. J. Pharm. Exp. Therap. 137: 249 (1962).

10. Wright, E. B. Action of Physostigmine (Eserine) Sulfate on Peripheral Nerve. Amer. J. of Physiol. 184:209 (1956)

11. Bullock, T. H., Grundfest, H., Nachmansohn D., Rothenberg, M.A. and Sterling, K. Effect of Di-isopropyl Fluorphosphate (DFP) on Action Potential and Choline Esterase of Nerve J. Neurophysiology.

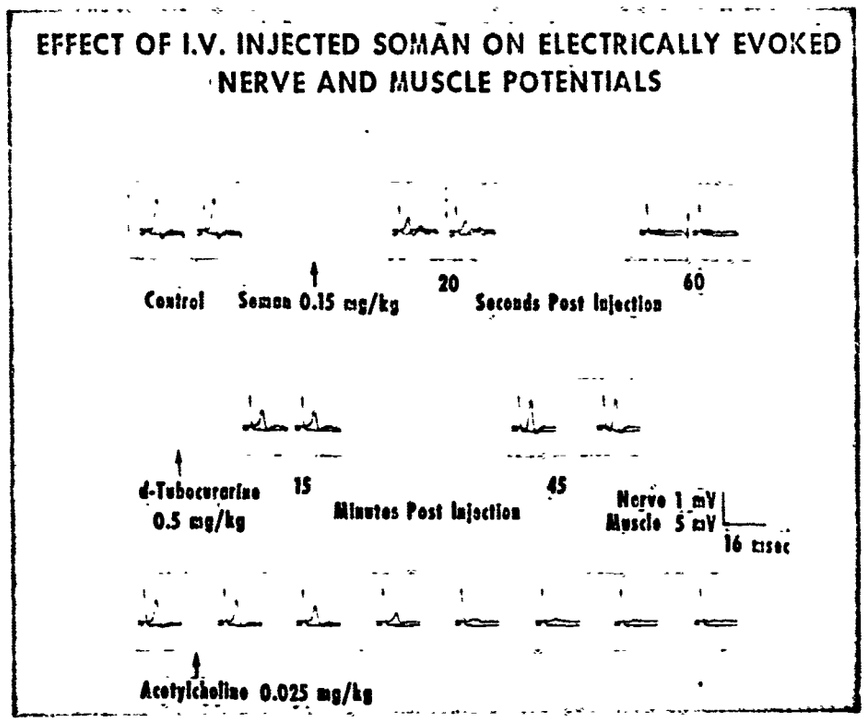

Figure I

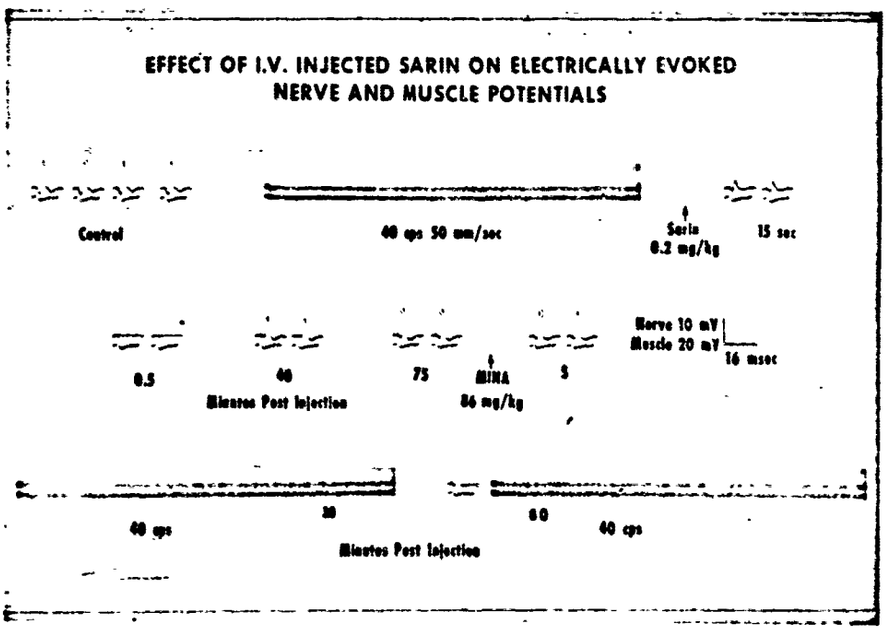

Figure 2

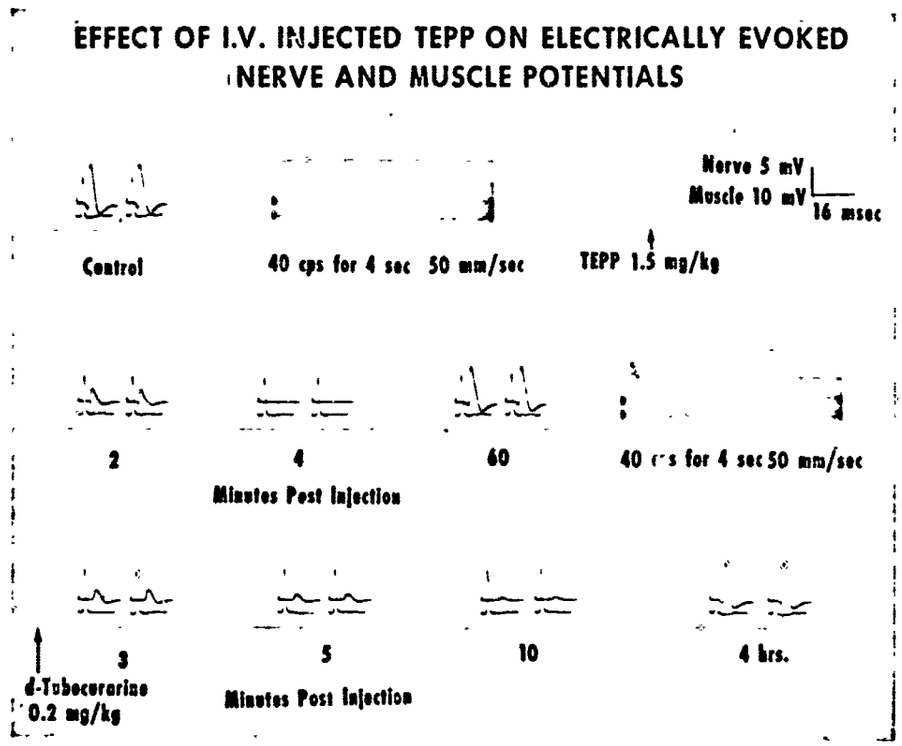

Figure 3

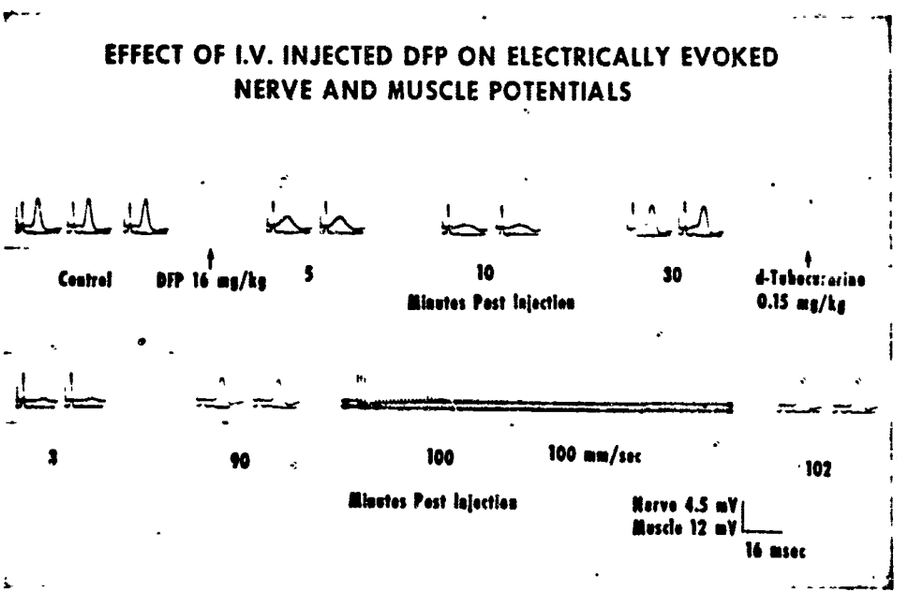

Figure 4

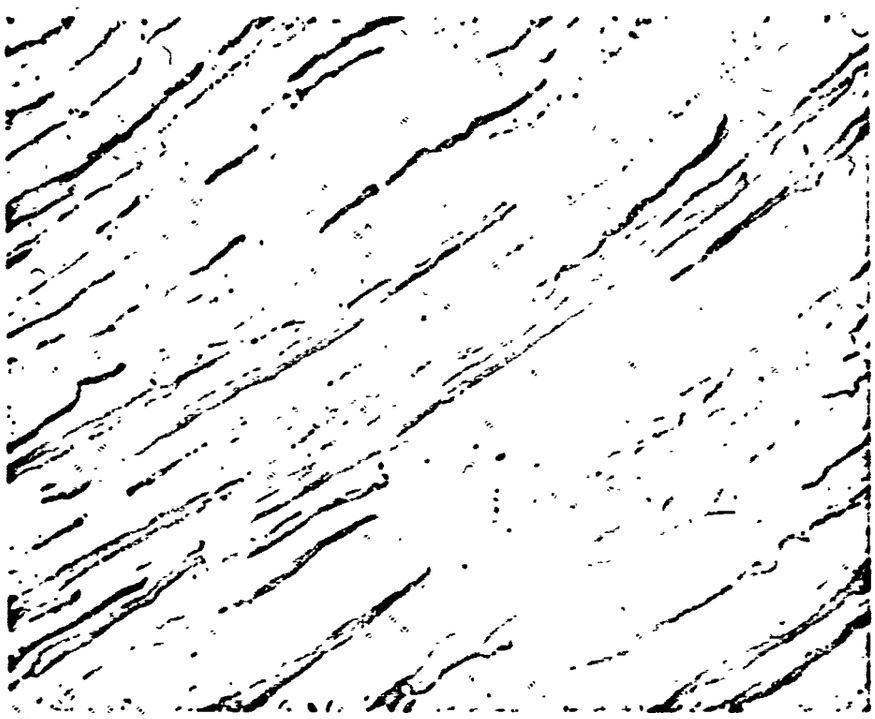

FIGURE 5. PHOTOMICROGRAPH OF LONGITUDINAL SECTION OF NORMAL SCIATIC OF CAT. STAINED HISTOCHEMICALLY FOR CHOLINESTERASE. THE CHOLINESTERASE APPEARS BLACK IN THE PHOTOMICROGRAPH. COUNTERSTAINED.

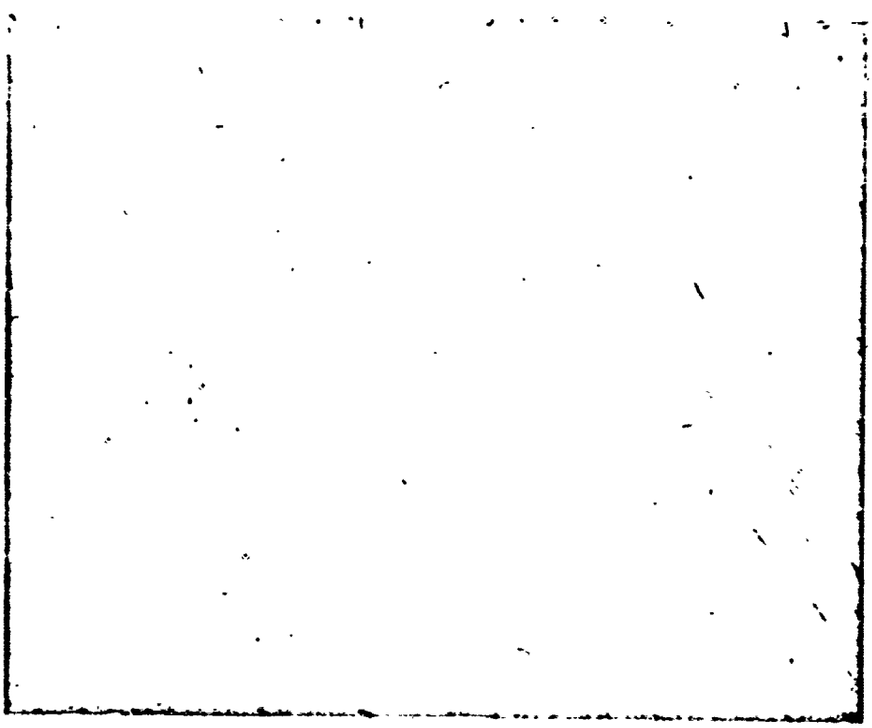

FIGURE 6. PHOTOMICROGRAPH OF LONGITUDINAL SECTION OF CAT SCIATIC NERVE AFTER SOMAN (0.15 MG/KG). STAINED HISTOCHEMICALLY FOR CHOLINESTERASE. THE CHOLINESTERASE APPEARS BLACK. COUNTERSTAINED.

FIGURE 7. PHOTOMICROGRAPH OF TRANSVERSE SECTION OF NORMAL SCIATIC NERVE OF CAT. STAINED HISTOCHEMICALLY FOR CHOLINESTERASE. THE CHOLIN ESTERASE APPEARS BLACK. COUNTERSTAINED.

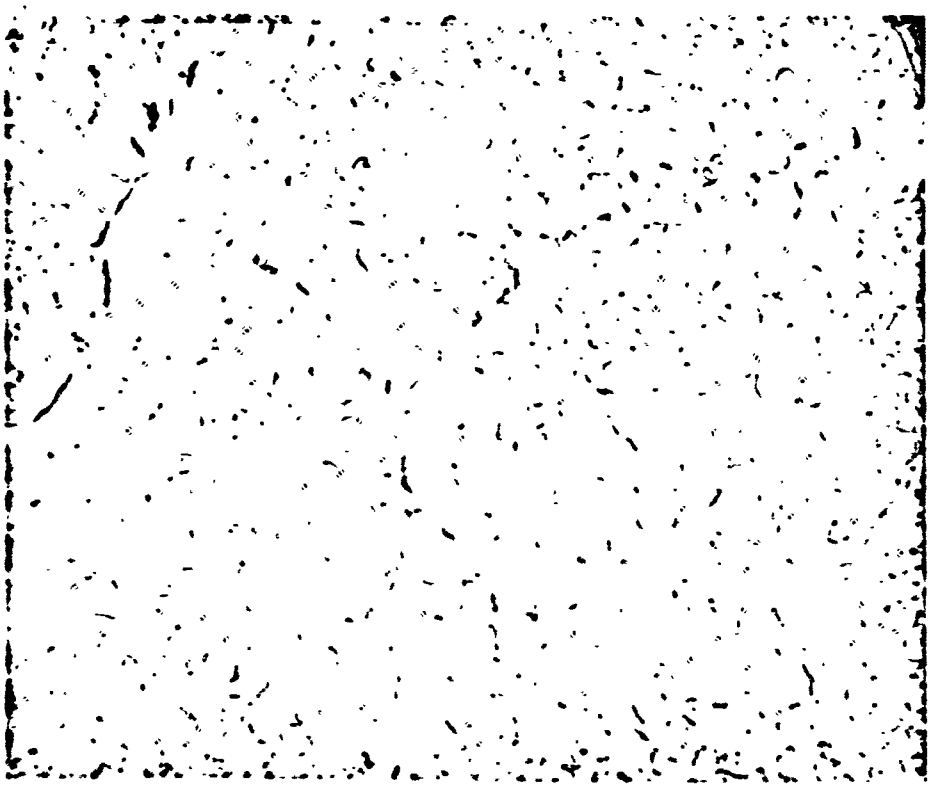

FIGURE 8. PHOTOMICROGRAPH OF TRANSVERSE SECTION OF CAT SCIATIC NERVE AFTER SOMAN (0.15 MG/KG). STAINED HISTOCHEMICALLY FOR CHOLINESTERASE THE CHOLINESTERASE APPEARS BLACK. COUNTERSTAINED.